SUPPLEMENTARY MATERIAL

Cytotoxic activity of butanolic extract from *Sambucus nigra* L. flowers *in natura* and vehiculated in micelles in bladder cancer cells and fibroblasts

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Bladder cancer has a high incidence and recurrence rate among patients worldwide. This study aimed to evaluate the cytotoxic activity of fractions of *Sambucus nigra* L. flower extracts on bladder carcinoma cells (T24 cells) and human fibroblast cells (MRC -5). The butanolic fraction (F-BuOH) was characterized by UPLC-DAD-MS/MS and nine flavonoids were identified. Rutin was the major compound. The cytotoxic activity of this fraction was observed in the T24 cells but not in MRC-5 cells, indicating selectivity. F-BuOH was incorporated in micellar solutions of Pluronic® F127 and cytotoxic effect for T24 cells was observed again. *In vitro* assay demonstrated a controlled release of the fraction from the micelles. The results obtained showed that flavonoids are the possible responsible for cytotoxic activity in bladder carcinoma cells. In addition, micellar solutions act together to increase the action of the butanolic fraction.

Keywords: *Sambucus nigra* L., flavonoids, cytotoxic activity, bladder cancer, polymeric micelles, Pluronic® F127.

Experimental section *Plant material*

Flowers (1kg) of *S. nigra* were obtained through Natural Amazon supplier, internal batch 0189-IF, batch manufacturer RSAB0001B6 / 14, quality control analysis certificate by Naturell Indústria e Comércio Ltda on June 30th, 2014. Flowers were crushed to the fine powder state in a knife mill (MR Manesco®, Brazil). The access was registered in the National System of Genetic Heritage Management and Associated Traditional Knowledge (SisGen), registration number A3E7C8F.

Crude ethanolic extract obtainment (EEB).

S. nigra flowers were extracted by 8 percolations (2000 mL ethanol 92.8°GL) at room temperature, with an average interval of 5 days of extraction between each percolation. The solvent was completely removed using a rotatory evaporator under reduced pressure at 50°C and then finally dried at 30-40°C and in desiccator. 373.03g of EEB were obtained, totaling a yield of 37.3%.

Fractionating the Crude Ethanolic Extract

EEB (15g) was solubilized with 200 mL of MeOH:H_2O (8:2) and subjected to successive liquid-liquid partitions in the separatory funnel using the solvents in the order: hexane, ethyl acetate and butanol (4 x 200 mL). The fractions obtained were ethyl acetate (F-AcOEt), butanolic (F-BuOH) and hydromethanolic (F-HMeOH). Fraction yields after the partition of the EEB were as follows: hydromethanolic (F-HMeOH) (46%), ethyl acetate fraction (F-AcOEt) (22%) and butanolic (F-BuOH) (8%).

Total phenolic compounds content

The quantification was performed following the methodology described by Seibert et al. (2019), and the results were expressed as EAG (gallic acid equivalents) per gram of sample (mg EAG/g).

Total flavonoid content

The quantification was performed using a colorimetric method of aluminum chloride ($AlCl_3$) (Seibert et al. 2019) and the results were expressed in EQ (quercetin equivalents) per gram of sample (mg EAQ/g).

Analyzes by ultra-high-performance liquid chromatography coupled to diode array detector and mass spectrometry (UPLC-DAD-MS/MS)

F-AcOEt, F-BuOH and F-HMeOH fractions were solubilized in MeOH: H_2O (97:3). The analyzes were carried out in UPLC ACQUITY equipment (Waters®), chromatographic HSS column (1.7 µm, 50 × 2 mm i.d.) (Waters). The sample injection volume was 4 µL, gradient elution with H_2O (0.1% formic acid) / Acetonotrile (ACN) (0.1% formic acid). The chromatographic parameter used comprised a linear elution period (5-95% ACN of 0 to 10 min), followed by a short isocratic elution period (95% ACN of 10 to 11 min), returning to the condition of the initial elution 11 to 13 min (5% ACN). The UV spectra were registered from 190 to 450 nm. The mass spectra were obtained with electron spray ionization and were recorded in full scan and sequential or tandem in the positive and/or negative modes in the Waters ACQUITY® TQD equipped with a quadrupole analyzer. The general conditions of operation of the equipment during the analyzes were capillary voltage: 3.5kV; capillary temperature: 320°C; desolvation temperature: 320°C; cone voltage: 5kV; ionization voltage: -4kV; hole voltage: -60kV. The samples were injected by automatic injection pump with continuous flow of 0.1 µL/min. ESI/MS/MS spectra were recorded with energy of 30eV in the range of *m/z* 100 to 1500 Da.

Preparation of micellar solutions by the cold dispersion method (DF)

The Pluronic® were added to a beaker containing ultrapure water, kept in an ice bath under moderate magnetic stirring until complete dispersion of the polymer. After 24 hours at 4ºC to allow the complete dissolution of the polymer, the butanolic fraction was added to the polymer solution and dispersed under magnetic stirring. Several combinations of different Pluronic® F127 and butanolic fraction concentrations were tested: FFB 10% (0.2g Pluronic® F127; 0.004g butanolic fraction; 1.79 mL ultrapure water), FFB 3% (0.06g Pluronic® F127; 0.008g butanolic

fraction; 1.93 mL ultrapure water), F127 10% (0.2g Pluronic® F127; 1.8 mL ultrapure water), F127 3% (0.06g Pluronic® F127; 1.94 mL ultrapure water).

Determination of particles size and zeta potential in formulations

The size distribution and the mean size of the micelles were determined using photon correlation spectroscopy, also known as dynamic light scattering using the Zetasizer (Malvern, Nano ZS model) equipment. Samples were diluted with ultrapure water and added in a quartz cuvette. The measurements were performed with incident spreading angle of 90º. Results were expressed as mean particle diameter and polydispersity index (PI). The zeta potential was determined by the electrophoretic mobility measurements of the suspended micelles. The equipment used, Zetasizer (Malvern, model Nano ZS), has a cell containing electrodes of opposite charges, one at each end of a U-shaped tube, where the diluted samples were added for analysis. The readings were performed at 25°C in triplicate, and the results were expressed in millivolts (mV).

Release of the butanolic fraction from the micelles

The *in vitro* release followed the method of Assis (2014) and used as a marker the flavonoid rutin. The equipment and the chromatographic cell used were Waters Alliance 2695 and Octadecyl-C18 Column, Phenomenex® (250, 0.1 mm x 4.6 mm x 5.0 μm) respectively. The sample injection volume was 20 μl, with gradient elution with H_2O (0.1% formic acid)/ACN (0.1% formic acid). The chromatographic parameter used was the same of UPLC-DAD-MS/MS with a time of elution of 30 min. Eluations were monitored in 220-400 nm. Each curve point sample was filtered with a Millex HV unit with Durapore® membrane 13 mm in diameter and 0.45 μm pore. The procedure was performed in triplicate and the mean areas of the peaks referring to the standard (rutin) were plotted as a function of the nominal concentrations (25 μg/mL, 10 μg/mL, 8 μg/mL, 5 μg/mL and 2 μg/mL) in a graph. In order to evaluate the efficiency of the analytical method, the linearity, precision, accuracy, limits of detection and quantification of each analytical curve were analyzed after the calculation of the linear correlation coefficient (r^2) (Nunes et al. 2005). This same curve

was used to determine the actual concentration of the biomarker. The calibration curve was redone for the solubility test and the *in vitro* release assay.

Solubility

The study of the sink condition was performed by evaluating the solubility test of the rutin flavonoid standard, in which 3.8mg of rutin (80% excess of the amount incorporated in the formulation) was added in 100 mL of the receiving solution, respecting conditions (triplicate). The solutions were shaken in an ultrasonic bath at 32.5°C for 1 hour. Samples were diluted to obtain the concentration of 19 μg/mL for injection in the HPLC and filtered in a HV Millex unit with a Durapore® membrane of 13 mm in diameter and pore 0.45 μm. Subsequently, the flavonoid was quantified (Rissi 2013).

In vitro release of FFB 3%.

The release of FFB 3% was performed by the regular dialysis method (Neckel and Lemos-Senna 2005). The procedure consisted of the use of dialysis bags, consisting of a dialysis tubing cellulose membrane (D9777-100FT, 25x16 mm - Sigma-Aldrich®, St. Louis, USA), in which each dialysis bag was attached to a glass rod at both ends; 1.0 ml of formulation was added in each. In a beaker, 200 mL of receiving medium (PBS-10x Phosphate Buffered Saline-Thermo Fisher Scientific®) was added, where glass rods containing the dialysis bags were also inserted. Dialysis was performed at 37°C and horizontal shaking, with withdrawals of 1.0 mL aliquots from the receiving medium and the volume removed from the medium replaced with equal amount of receiving solution, keeping the system under the same working conditions. The aliquot of the receptor medium was analyzed as the percentage of the actual amount of F-BuOH fraction released through the biomarker rutin capable of overcoming the dialysis membrane. The collection times were: 0, 1, 2, 3, 4, 5, 6, 12 and 24 hours; the formulation submitted to the release test was FFB 3%. The quantification of the fraction was determined by HPLC-DAD, using as biomarker the rutin flavonoid.

Evaluation of cytotoxic activity

Human urothelial carcinoma cells T24 (high grade, invasive) and human lung fibroblast cells MRC-5 were used in the cytotoxicity assay. Both cells were maintained in Dulbecco's modified Eagle culture medium (DMEM), supplemented with 10% fetal bovine serum, 100 U/mL penicillin G, 100 U/mL streptomycin and maintained at 37°C in 5% CO_2 atmosphere. The cytotoxicity assay was performed using the Cell Proliferation II Kit (XTT, Roche). Cells were seeded in 96-well culture plates (1×10^4 cells/well) and after 24 hours treated with the F-AcOET, F-BuOH and F-HMeOH fractions (2 μg/mL, 5 μg/mL, 10 μg/mL, 15 μg/mL, 20 μg/mL, 50 μg/mL, 100 μg/mL, 200 μg/mL, 400 μg/mL, 600 μg/mL, 800 μg/mL, 1000 μg/mL). After incubation for 24 hours, the cells were washed with Hank's solution (0.4g KCl, 0.06g KH_2PO_4, 0.04g Na_2HPO_4, 0.35g $NaHCO_3$, 1g glucose and 8g NaCl in 1 L of water). Afterwards, white culture medium (DMEM without phenol red) was added to the wells and then 12 μL of the XTT test solution (50 parts XTT labeling solution: 1 part electron-coupling reagent) was added. The absorbance was measured at 492 and 690 nm after 60 min. Absorbance results are proportional to the number of viable cells. The tests were conducted in triplicate. The calculation used was: % of viable cells = (mean of the absorbances/mean of the absorbances of the control) * 100 (Sávio et al. 2014). The same procedure was performed with the formulations FFB 10%, FFB 3%, F127 10% and F127 3% (25 μg/mL, 50 μg/mL, 100 μg/mL, 200 μg/mL, 400 μg/mL, 600 μg/mL) and pure standard of rutin flavonoid (2 μg/mL, 4 μg/mL, 6 μg/mL, 8 μg/mL and 10 μg/mL) (Srivastava et al. 2016). Untreated cells and micellar solutions of Pluronic® F127 without the incorporated butanolic fraction were used as controls. IC_{50} values at concentrations of 2 μg/mL to 1200 μg/mL were calculated. The selectivity index (SI) of the fractions was calculated from the ratio of the IC_{50} values found for normal cells (MRC-5) on the IC_{50} values of tumor cells (T24).

Statistical analysis

For the statistical analysis, the GraphPad Prism 5 software was used. As the data of the results of the tests of cytotoxicity, phenolic dosage, flavonoid dosage and *in vitro* release of the butanolic

fraction presented normal distribution, ANOVA followed by the test of Tukey's multiple comparison was realized. The P value < 0.05 was considered statistically significant.

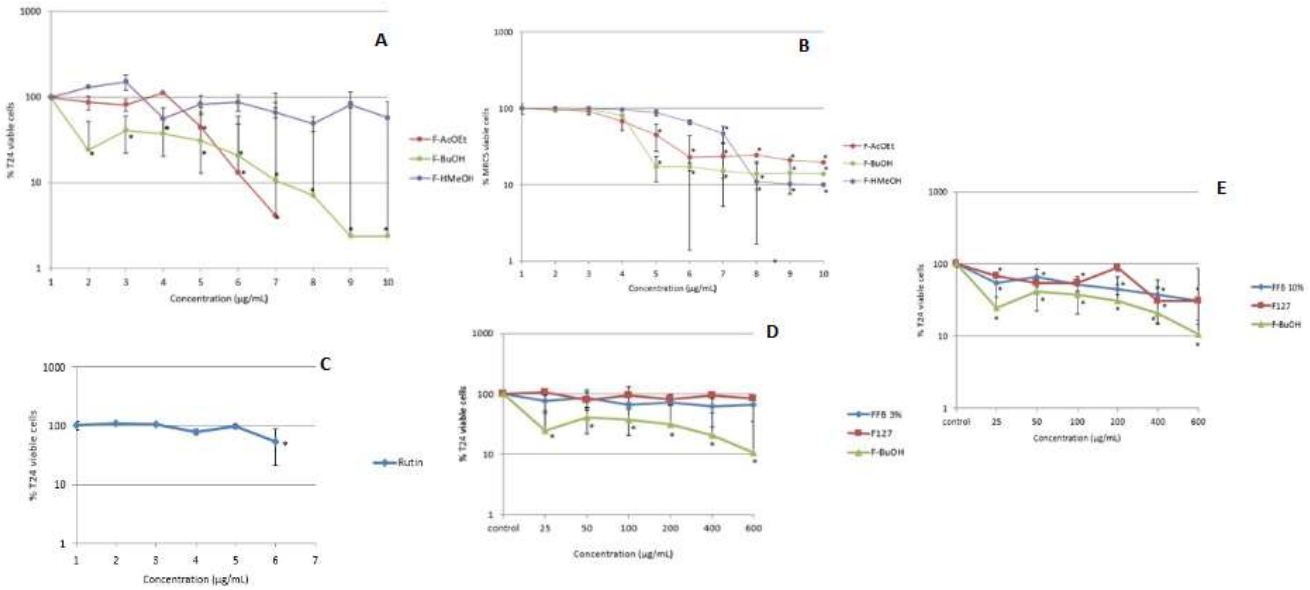

Figure S1 A. Percentage of viable T24 cells 24 hours after treatment with the F-AcOEt, F-BuOH and F-HMeOH fractions. **B.** Percentage of viable MRC-5 cells 24 hours after treatment the F-AcOEt, F-BuOH and F-HMeOH fractions. **C.** Percentage of viable T24 cells 24 hours after treatment with pure rutin standard. **D.** Percentage of viable T24 cells 24 hours after treatment with FFB 3%. **E.** Percentage of viable T24 cells 24 hours after treatment with FFB 10%. Each point represents the mean obtained in three experiments. * Significant difference p <0.05

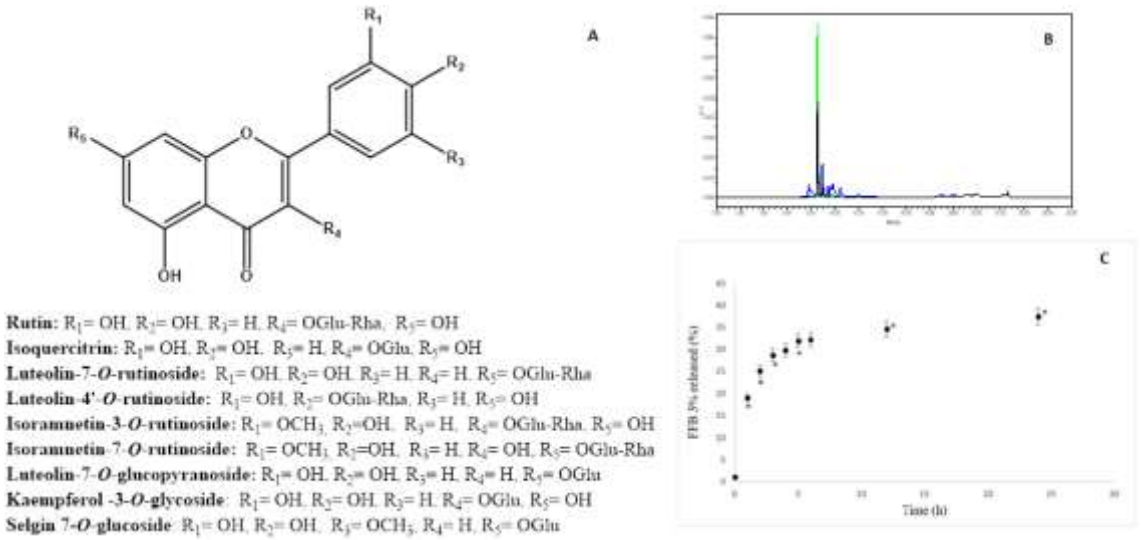

Figure S2 A. Chemical structures of the main flavonoids identified in *Sambucus nigra* L **B**. Overlapping of the chromatogram of the F-BuOH fraction (blue) with the chromatogram of the rutin standard (green) obtained by HPLC-DAD. **C**. Percentage of 3% FFB released over time using the rutin flavonoid as biomarker.

Table S1 IC_{50} values for human lung fibroblast cells (MRC-5) and urothelial carcinoma cells (T24) and their respective selectivity indices (SI).

	MRC-5 IC_{50}	**T24 IC_{50}**	**SI**
	µg/mL		
F-BuOH	418,4	21,57	19,39
F-AcOEt	230,2	209,5	1,09
F-HMeOH	575,84	1217,7	0,47

IC_{50} = Concentration capable of inhibiting the number of cells in culture by 50%.
SI = IC_{50} MRC-5 / IC_{50} T24

Table S2 Total phenolic compound content and total flavonoid content for the AcOEt, BuOH and HMeOH fractions.

	Total Phenolic Compound Content	**Total Flavonoid Content**
	mg EAG/g	mg EQ/g
F-BuOH	54.87 ± 0.83	16.16 ± 0.75
F-AcOEt	45.00 ± 1.18	6.89±0.48
F-HMeOH	23.08 ± 1.56	4.15 ± 0.25

Results were presented as mean ± standard deviation.

Table S3 Maximum absorption in UV / Vis (λmax - nm) and fragments generated (MS/MS - *m/z*), in negative mode, for the compounds identified in the butanolic fraction (F - BuOH) of the flower extract of *S. nigra*.

Substance	**Retetion time (min)**	**UV/VIS $\lambda_{máx}$ (nm)**	**$[M-H]^-$ (*m/z*)**	**EM/EM (*m/z*)**	**References**
Rutin	2.70	255; 354	609.91	300.94; 179.22	Zhang et al. 2010
Isoquercitrin	2.84	255; 354	463.46	300.20	Tiberti et al. 2007
Luteolin-7-*O*-rutinoside	3.02	265; 331	593.50	285.00	Ola et al. 2009; Plazoni et al. 2009
Luteolin-4'-*O*-rutinoside	3.02	265; 332	593.63	284.87	Ola et al. 2009
Isoramnetin-3-*O*-rutinoside	3.09	253; 345	623.76	315.12	Willems and Low 2018
Isoramnetin-7-*O*-rutinoside	3.09	253; 345	623.89	315.24	Truchado et al. 2009
Luteolin-7-*O*-glucopyranoside	3.11	265; 332	447.26	284.07	Plazoni et al. 2009
Kaempferol -3-*O*-glycoside	3.11	253; 344	447.26	283.88	Bao et al. 2018
Selgin 7-*O*-glucoside	3.19	253; 340	477.30	313.83	Marin et al. 2004

Table S4 Average size, polydispersity index (PI) and zeta potential of formulations obtained by the cold dispersion method (DF).

Formulations	Average size ± dp (nm)	PI ± dp	Zeta potential ± dp (mV)
FFB 10%	180,8±16,54	0,558±0,14	-29,5±2,93
FFB 3%	259,6±20,85	0,704±0,142	-22±3,3
F127 10%	72,03±40,42	0,186±0,011	-43,2±2,77
F127 3%	247,3±76,33	0,528±0,155	-34,6±2,55

FFB 10% (2mg/mL Formulation of Butanolic Fraction containing 10% Pluronic® F127); FFB 3% (3mg/mL Butanolic Fraction Formulation containing 3 % Pluronic® F127); F127 10% (10% of Pluronic® F127); F127 3% (3% of Pluronic® F127)

Table S5 Validation parameters for rutin quantification.

	Rutin (µg/mL)	Standart Deviation	Coefficient of Variation %
Linearity	2	125	0,245
	5	602	0,569
	8	880,52	0,468
	10	1112	0,401
	25	1870,50	0,313

	Repetitions	Rutin (8 µg/mL)	Average
Intermediate Precision	1	7.6503	7.6752
	2	7.7216	**Standart Deviation**
	3	7.6716	0.0388
	4	7.7239	**Coefficient of Variation %**
	5	7.6503	0.5055
	6	7.6333	

	Sampling (µg/mL)	Average Recovery %	Standart Deviation
Precision Accuracy	2 (low)	98.91	0.259
	8 (medium)	96.01	0.457
	25 (high)	98.99	0.179

Detection Limit	0.11 µg / mL
Quantification Limit	0.38 µg / mL

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